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Award Number: DAMD17-99-IA-9382

TITLE: Cell and Molecular Biology of Ataxia Telangiectasia Heterozygous Human Mammary Epithelial Cells Irradiated in Culture

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REPORT DATE: September 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2001	Annual (1 Sep 00 - 31 Aug 01)

4. TITLE AND SUBTITLE
Cell and Molecular Biology of Ataxia Telangiectasia Heterozygous Human Mammary Epithelial Cells Irradiated in Culture

5. FUNDING NUMBERS
DAMD17-99-IA-9382

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Autologous isolates of cell types from obligate heterozygotes with the autosomal disorder ataxia-telangiectasia (A-T)were used to begin a tissue culture model for assessing pathways of radiation-induced cancer formation in this target tissue. This was done by establishing cultures of stromal fibroblasts and long-term growth human mammary epithelial cells (HMEC) in standard 2-dimensional tissue culture in order to establish expression of markers detailing early steps of carcinogenisis. The presumtive breast cancer susceptibility of A-T heterozygotes as a sequael to damage caused by ionizing radiation provided reason to study expression of markers in irradiated HMEC. Findings from our study with HMEC have included determination of differences in specific protein expression amongst growth phase (e.g., log vs stationary) and growth progression (e.g., pass 7 vs pass 9), as well as differences in morphologic markers within populations of irradiated HMEC (e.g., development of multinucleated cells).

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	7
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Individuals who carry two defective genes for the syndrome ataxia-telangiectasia (A-T) are prone to a number of clinical pathologies that probably relate to the lowered ability to repair DNA and chromosome damage in their cells. This damage can be either naturally created internally, such as by the normal rearrangement of DNA in immune cells responding to challenge by antigens, or externally created, such as by the local energy deposited by ionizing radiation. One long-term consequence of this lowered repair is a high rate of malignant cancers such that lymphomas and leukemias, for example, occur more than a hundred times more often in those with two defective genes than in those with nondefective genes.

There are only about 600 people in the United States with two defective genes for A-T, but about 1.5% of the entire population carry a single defective gene. It is important to ask, then, what does a single defective A-T gene do in terms of cell biology in this rather large number of carriers who are not acutely affected, and who indeed appear clinically normal? Furthermore, there is putative evidence that genetic damage caused by ionizing radiation contributes to the observed 5- to 7-fold increased risk of breast cancer for female A-T heterozygotes in the national population. Therefore, it is important to ask also, what does A-T heterozygosity do in response to radiation damage that contributes to the phenotypic epression of breast cancer specifically?

Three experimental Aims were proposed. First, using A-T carrier breast-derived cells, establish the relationship amongst radiation-induced expression of genes believed to be involved in the molecular biology of cancer formation. Second, extend the same radiation studies to these A-T carrier breast cells that have additionally been genetically altered in ways that establish built-in steps in the known cancer producing pathway. Third, acquire additional specimens of breast tissue from A-T carrier women so that more cell isolates can be studied in replicate experiments and thereby provide statistical significance to the experimental findings.

From these Aims, specific knowledge may be gained for use in understanding, and thereby influencing, breast cancer susceptibility in female carriers of the defective A-T gene.

BODY

Ataxia-telangiectasia (A-T) is a radiation-sensitive autosomal genetic syndrome that broadly poses two levels of risk to the recipients of the A-T gene located on chromosome 11q22-23, that is, to those who are homozygous recessive (and clinically defined) and to those who are heterozygous (and clinically silent) for this gene. It is hypothesized that the known radiation sensitivity of A-T homozygous cells will be reflected to some degree in the radiation responses established for A-T heterozygous human mammary epithelial cells (HMEC), and that this sensitivity will effect expression of carcinogenic process responsible for the known breast cancer susceptibility in women who are heterozygous for the A-T gene.

Experimental work designed to investigate the underlying mechanisms of carcinogenesis has been the central focus of Year 2. This work is described below.

WORK RESPONDING TO SPECIFIC AIMS 1, 2, and 3

1. Markers Expressed by A-T Heterozygous HMEC

a. Immunogenic Markers in Nonirradiated Cells in Tissue Culture

Both HMEC and autologous fibroblasts isolated from the breast tissue samples produced copious extracellular matrix in culture. The dense matrix associated with attached HMEC and fibroblasts necessitated development of stringent sample preparation in order to eliminate false negative staining caused by retention of primary and secondary anitbody used in the two-step antibody-biotin complex method of immunogenic staining for biochemical markers of expression in these cells.

False negative antibody staining of these attached mammary cells was found to be a problem in Year 1, and that high detergent concentrations in the wash buffer helped to provide satisfactory results. During Year 2 we invested additional effort comparing wash buffer containing 1% vs. 3% Tween 20, and the DAB immunostaining step being done in 1% Tween 20 buffer vs. water alone, with and without initial blocking of both fresh and fixed cells, and compared different suppliers of mouse IgG used for negative controls. Because culture dishes and plates can be generated faster than the ability to keep up with immunostaining, we also compared results from cultures stored in a variety of ways for different lengths of time prior to immunostaining and compared outcome with that from freshly fixed cells.

We determined: that no sample blocking is better than the use of any one of several blocking agents (bovine serum albumin, horse serum, fetal bovine serum, Pierce Superblock, powdered milk); that all immunostatining solutions containing 1% Tween 20 detergent produced superior low frequency of false negative controls (run with every assay); that Jackson Labs mouse IgG (compared to Sigma and to Vector) was superior for providing low frequency of false negative controls; that storage of fixed 96-well plates of cells retained good immunogenic staining for at least 3 months although cellular morphology lost some definition over that time period. It was determined that fibronectin was expressed heavily in cells at all times, and this marker was instituted for the generic positive control carried with all immunostaining experiments.

Having established stringent controls and procedure for immunostaining, we proceeded to analyze a range of protein expression markers in cells grown in 96-well plates. Distinguishing protein expression based upon growth phase of cells (log vs. stationary), cell age (passage number), cell type (fibroblasts vs. HMEC preparatory to coculture studies), and extracellular matrix components. Among the markers surveyed, expression of collagen I, collagen IV, collagen VII, connexin, and estrogen receptor were distinguished between HMEC and fibroblasts. Collagen IV, collagen VII, connexin, and estrogen receptor were positive for HMEC, while collagen I was positive for fibroblasts. Cytokeratins 18 and 14, and alpha-actin were positive in HMEC. Common expression of this latter combination suggests that these long-term growth-selected isolates have dedifferentiated to express markers characteristic of luminal HMEC (cytokeratin 18), basal HMEC (cytokeratin 14), and myoepithelial HMEC (alpha-actin).

We examined protein markers in HMEC as a function of growth phase (log vs stationary), cell age (passage number 7 through 10), and appearance of specific proteins known to potentially reflect and/or influence transformation pathways. Cytokeratins 18 and 14 were heavily expressed in stationary phase HMEC, but not log phase. Estrogen receptor, conversely, was expressed heavily in log phase HMEC but not sationary phase. Laminin was noted to extend extensively into extracellular matrix from log phase HMEC, but not Collagens IV or VII, and not chondroiton sulfate or fibronectin. E-cadherin was not observed at any time by immunostatining, and neither was her-2/neu receptor.

b. Immunogenic and Morphologic Markers in Irradiated Cells in Tissue Culture

HMEC were prepared into four late log-phase T25 flasks. One flask served as a 0 rad control and one each of the remaining three flasks were irradiated with 30 rad, 60 rad, and 90 rad of Cobalt-60 gamma-radiation, respectively.

Cells irradiated at pass 7 in T25 flasks were split into 96-well plates for immunogenic staining, and were also passed further within T25 flasks and inspected for development of morphologic markers of radiation damage. Retinoic acid was also added to a series of 96-well samples in order to test for effects of this differentiating agent on expression of protein markers examined. The immunostaining of these irradiated cells are still underway at this time.

The morphologic markers were most obvious in the postirradiation appearance of multinucleated cells. A 2% appearance of binucleated cells in pass 11 of nonirradiated cells increased dramatically to 20% in cells irradiated with 90 rad..

2. Work With A-T Heterozygous Transformed HMEC

Transformed HMEC had been acquired by the beginning of the grant period. These cells had been presumably immortalized by selection of extended growth populations of A-T heterozygous HMEC infected by HPV type E6 and E7 oncogenes in the laboratory of Dr. Ray White at the University of Utah.

These cell lines were not examined in Year 2, but will be entered into study during Year 3.

3. Tissue Harvest and Cell Isolation

In February of 2000 a second breast tissue harvest was collected and from an obligate A-T heterozygote and processed by the PI. The surgery providing this specimen was identified by Dr. Mike Swift, as had been done in the previous instance that led to the PI establish a repository of A-T heterozygous HMEC and autologous cells types that in turn formed the foundation of proposed research for this grant. This second specimen was obtained from a 32 year-old white female FT as a single mastectomy from which approximately 25% of initial tissue had been removed for use by surgical pathology. The specimen was anticipated to be noncancerous, and indeed the patient had elected prophylactic mastectomy at the time of her operation scheduled for removal of the contralateral breast known to contain cancer. However, the prophylactic specimen was subsequently determined to have central regions of infiltrating globular ductal carcinoma as well as ductal carcinoma in situ. Harvest of this tissue has to date led to isolation of organoids, overlying skin fibroblasts, and stromal fibroblasts. The isolation of LTG HMEC from those organoids were planned late in Year 2, to be done in similar fashion with procedure used for establishing the repository from the first, patient KW.

At the time of submission of this proposal, Dr. Mike Swift was committed to provide efforts to connect Dr. Richmond, PI with A-T heterozygote-related individuals from his extensive database who were scheduled to undergo mastectomies for medical purposes. This was a supportive contribution from Dr. Swift, i.e., he did not receive budgeted support. From those patients and their attending surgical staff, the PI was then going to arrange for donation of discard breast tissue from which to harvest additional autologous cells to enter into study. Tissue from the patient FT of February 2000 was acquired by that plan, for example. Since then, however, funding provided for operation of Dr. Swift's database during Year 2 has been inadequate for him to canvass the approximately 10,000 individuals to identify A-T heterozygote-related surgical cases. For this reason, Year 2 has not provided initially anticipated additional breast tissue acquisitions. The PI will seek additional tissue acquisitions from Dr. Swift's database during Year 3.

KEY RESEARCH ACCOMPLISHMENTS

1. It is thought that novel observations have been made in terms of patterns of marker expression in HMEC as a function phase of growth, age of cells, and expression of specific markers.
2. It is thought that novel observation has been made with the observation of radiation-induced multinucleation in HMEC populations.
3. Year 2 progress covering September 30, 200 through September 29, 2001, included the following advances in the infrastructure of this project.
    a. hiring a postdoctoral fellow under separate mechanism with start date August 1 of 2001 to work on these Aims
    b. advancing the use of a Becton-Dickenson FACSCalibur flow cytometer purchased under separate mechanism in August of 2000 for application to these Aims
    c. initiating isolation and repository of a second A-T heterozygote breast tissue harvest obtained in February of 2000

REPORTABLE OUTCOME

1. No publications resulted in this reporting period (one is in preparation).
2. One report on this work was presented at the Cell Science Meeting of the NASA Cell Science Program at Johnson Space Center in March, but this meeting did not produce proceedings.

CONCLUSIONS

Repositories of cells isolated from additional A-T heterozygous breast tissue need to be established for intercomparisons.

Study of expression of markers of related to HMEC function and differentiation is yielding a potentially valuable database for extending to a model determining effects of ionizing radiation that will assist in risk assessment of cancer caused by ionizing radiation. These markers are of intensity and of variation such that flow cytometric determination additionally will prove valuable in quantitating appearance of markers and their response and regulation to treatments involving ionizing radiation.